November 14, 1995

Food and Drug Administration
Animal Drugs Research Center
Denver Federal Center
P.O. Box 25087
Denver, CO 80225-0087

A. TITLE

Determination of Malachite Green and Metabolite, Leucomalachite Green in Catfish (Ictalurus punctatus) Tissue by High Pressure Liquid Chromatography with Visible Detection (LC/VIS).

JOSÉ E. ROYBAL, Allen P. Pfenning, Robert K. Munns, David C. Holland, Jeffrey A. Hurlbut and Austin R. Long.

B. SCOPE

Malachite Green (MG) is a cationic triphenylmethane dye commercially available as the oxalate and hydrochloride salts (1). MG is a topical antiseptic which has been used in fisheries and the aquaculture industry as a fungicide. MG is used to control the growth of fungi on fish and incubating fish eggs. It is extremely effective when used as a flush treatment at 1.0 ppm alone or in combination with 20 ppm formalin (2). Clifton-Hadley and Alderman (1987) reported that MG deposits rapidly in rainbow trout exposed to flush treatments and that repeated treatments were cumulative (3). Bauer, K., et al, stated that trout excreted the intact MG rapidly, but that the leuco base, Leucomalachite Green (LMG), stored in the muscle tissue had a long residence time, with a half-life of approximately 40 days (4).

Some triphenylmethane dyes, including MG, have been recognized as animal carcinogens (6-8). Potential MG residue carryover resulting from its use in the aquaculture industry establishes the need for specific and sensitive analytical methods which will allow us to monitor MG use in the aquaculture and seafood industries. Although methods exist for the analysis of MG in water (5), little has been published on the analysis for MG in tissue matrices(4,9-10). This method isolates and quantifies the levels of MG and its LMG metabolite in catfish muscle.

C. PRINCIPLE

The analytes are extracted from tissue with acetonitrile/buffer and partitioned into methylene chloride. Final cleanup and isolation is performed on neutral alumina SPE and propylsulfonic acid cation exchange SPE columns prior to LC analysis. Liquid chromatography is performed by isocratic elution with a buffered mobile phase from a cyano column. Post-column oxidation using a PbO_2 post-column reactor coupled with visible detection (LC/VIS) is used for analysis. Recoveries of MG from fortified catfish tissue were 72.9%, 1.92%RSD (23 ppb) , 75.5%, 6.85 %RSD (11 ppb), and 69.6%, 6.93%RSD (5.7 ppb). Recoveries of LMG from fortified catfish tissue were 87.4%, 2.92%RSD (21 ppb), 88.1%, 5.94%RSD (10 ppb), and 82.6%, 11.5%RSD (5.3 ppb).

D. MATERIALS

1. Apparatus

(a) Pasteur pipet.- Disposable, 5.75 in.

(b) Centrifuge tube.- 200mL, Falcon Blue Max, disposable, conical, graduated, polypropylene with cap (cat.# 2075, Becton/Dickinson, Lincoln Park,NJ 07035) and cushions (cat.# 2090, Becton/Dickinson) or equivalent.

(c) Centrifuge tube.- 15mL, graduated,with glass stopper #13 (Kimble cat. no. 45153-A). Optional: 15mL, Falcon Blue Max, disposable, conical, graduated, polypropylene with cap (cat.# 2096, Becton/Dickinson, Lincoln Park,NJ 07035) .

(d) Rotary evaporator.- Büchi Model R-110 with ice trap, evaporation temperature 50°C (Brinkmann Instruments, Inc. Westbury, NY 11590) or equivalent.

(e) Boiling flask.- 300mL, pear-shaped, acetylation, shortneck, with glass stopper s/t 24/40 (K-608700-0524, Kontes Glass Co.) or equivalent.

(f) Alumina (ALN-SPE) columns.- 6-mL HC Alumina-Solid phase extraction, 1000mg, neutral, P/N 7214-07 (JT Baker, Phillipsburg, NJ 08865) or equivalent, including Accubond neutral alumina (J&W Scientific).

(g) Propylsulfonic acid (PRS-SPE) columns.- Propylsulfonic acid-Solid phase extraction column, 500mg, Bond Elut LRC, P/N 1211-3038, Analytichem International/ Varian, (Varian, 24201 Frampton Ave., Harbor City, CA 90710) or equivalent.

(h) Column connection adaptors.- 12,20mL adaptor for PRS-SPE, Bond Elut LRC extraction columns (g), P/N 1213-1003, Analytichem International/Varian, (Varian, 24201 Frampton Ave., Harbor City, CA 90710) or equivalent.

(i) Liquid chromatograph system.- Shimadzu, Solvent Delivery Module, model LC-600 (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan), Rheodyne 100 µL fixed volume LC injector (Rheodyne Corp., Cotati, CA 94931).

(j) PbO_2 Oxidation column. - HPLC column, empty, stainless steel, 3cm length x 4mm id., inverted female end fittings with 2µ frit filters, (cat. # 184001, ES Industries, Marlton, NJ 08053) or equivalent.

(k) LC column.-Phenomenex Ultremex 5CN (cyano), 5 micron, 150mm x 4.6mm id. (Cat.# 00F-0050-EO. Phenomenex, Torrance,CA) or equivalent.

(l) UV/VIS detector.- Shimadzu Ultraviolet-Visible spectrophotometric detector, SPD-6AV module for HPLC (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) or equivalent,

(m) Integrator.- Hewlett-Packard Series II Integrator, Model HP3396B (Hewlett-Packard, HP Analytical Direct, Kennet Square, PA 19348) or equivalent.

(o) Homogenizer.- Tissuemizer, Model SDT1810 with Model 18N shaft (Tekmar Co., Cinninnati, OH 45222-1856) or equivalent.

(p) Beakers.- 600mL, Kimax 14000, Griffin low form with lip, pouring spout and capacity scale (cat.# 5330, ACE Glass, Inc., Vineland, NJ 08360) or equivalent.

(q) Funnel.- Separatory, 250mL, Squibb, Pear-shaped, with Teflon plug stopcock, Kimax 29048F (cat.# 7227-T, ACE Glass, Inc., Vineland, NJ 08360) or equivalent.

(r) Syringe.- 250 µL, Hamilton Model 725 (Baxter/Scientific Products, 1430 Waukegan Park, IL 60085), or equivalent.

(s) Filter paper.- Whatman No.1 or Schliecher & Schuell 597.

(t) Column heater.- Eppendorf or equivalent (this is recommended, but not required).

2. Reagents

(a) Solvents.- Distilled-in-glass, pesticide-grade, methylene chloride, methanol, UV spectro-grade acetonitrile (Burdick & Jackson, Muskegon, MI 49442) or equivalent.

(b) Water.- HPLC grade (Fisher Scientific ONLY. Other sources of water have been found to reduce recovery). Use for all subsequent references to water.

(c) Acetic acid.- ACS grade, glacial, aldehyde-free.

(d) Sodium acetate.- ACS grade, anhydrous.

(e) Lead Dioxide.- ACS grade, min. 95% PbO_2 powder, CAS# 1309-60-0 (cat.# 5727, Mallinckrodt, Paris, KT 40361) or equivalent. If lead oxide is grainy rather than powdery, it must be ground with a mortar and pestle to increase the surface area.

(f) Diethylene glycol (DEG).- ACS grade, 2,2'-Oxydiethanol, CAS 111-46-6.

(g) Alumina.- Alcoa type CG-20, 80-200 mesh, activated, chromatographic grade, CAS 1344-28-1 (cat.# AX0612-1, EM Science, Cherry Hill, NJ 08034) or equivalent. Recovery has been found to vary depending on the source of the alumina.

(h) Hydroxylamine Hydrochloride (HAH).- ACS grade, Oxammonium hydrochloride, CAS 5470-11-1, crystalline, grade I, 99%. (1) Dissolve 25g in 100 mL water (0.25 g/mL).

(i) p-Toluene sulfonic acid (p-TSA).- monohydrate, FW 190.2, ACS grade. (1) 1M p-TSA 19.0g/100mL water. (2) 0.05M p-TSA. Aliquot 5mL of i(1) to 100mL graduated cylinder. Dilute to 100mL with water.

(j) Hydroxylamine Hydrochloride/Methanol/p-TSA, 2.5mg/mL. - Aliquot 1mL HAH, (h(1)), and 10mL 0.05M p-TSA, (i(2)), into 100mL graduate cylinder. Dilute to 100mL with methanol, stopper and mix.

(k) Acetate buffer.- prepare by adjusting 1L of a 0.1M sodium acetate solution (8.2g sodium acetate/1000mL water) to pH 4.5 with acetic acid (ca 8mL), then add 5mL of 1M p-toluene sulfonic acid (i(1)).

(l) Mobile phase.- Acetonitrile/Acetate buffer (k) (45+55). It is necessary to oxidize any trace amounts of reducing agents in the mobile phase. Such contaminants may cause rapid consumption of PbO_2 in the post-column oxidizing chamber. After the mobile phase is prepared, add approximately 3g of PbO_2 per liter mobile phase and stir. Allow to sit approximately 10 min. Filter treated mobile phase through S&S 597 filter paper or equivalent. Finally, filter this solution for LC use through 0.45µm, PTFE membrane filter with appropriate apparatus.

(m) Celite.- Acid-washed, 545 (Johns-Manville, Denver, CO 80217) or equivalent.

(n) Reference Standards.- Malachite Green, 99%(CI 42000) as the oxalate salt (FW = 927.02), cat.# 1264, Eastman Kodak Company, Rochester, NY 14650. Leucomalachite green, CAS# 129-73-7, cat# L-7257, Sigma Chemical Co., St Louis, MO 63178.

mg(MG+) = Weight(mg) of MG Oxalate x (FW(MG+) x 2) / FW(MG Oxalate)

which is equivalent to:

mg(MG+) = Weight(mg) of MG Oxalate x 658.90 / 927.02

(o) Stock solutions (100ug/mL).- Accurately weigh 10.0mg of LMG standard and 14.0mg MG oxalate (calculate MG+ concentration after correcting for oxalate) into separate 100mL volumetric flasks, dilute to volume with methanol and mix.

(p) Fortification Standard Mix (1ug each/mL).- Pipet 0.1mL (100µL) of each stock solution into 10mL volumetric flask, dilute to volume with methanol and mix. Transfer to polypropylene tube for storage. Prepare daily. This solution tends to lose color after a few hours; this is acceptable.

(q) Mixed LC working solutions.- Prepare a series of mixed LC working standards at concentrations of 2.5, 6.25, 12.5, 25, and 50 ng each/mL. Pipet 10, 25, 50, 100, and 200µL of fortification standard mix (p) into separate 15mL centrifuge tubes. Add 2 mL mobile phase. Add 1mL HAH/MeOH-TSA ((j), 2.5mg/mL). Dilute to 4 mL with water, stopper and mix thoroughly. Based on 10g sample weights, solutions are equivalent to 1, 2.5, 5, 10, and 20 ppb each of MG and LMG. Prepare daily.

(r) PbO_2 Post Column Reactor. - Prepare a homogenous mixture, 10% by weight of lead dioxide with Celite. Seal one end of empty column with frit. Draw dry 10% PbO_2 mixture into other end of column using vacuum. Pack tightly by adding a few drops of MeOH. Repeat until column is full. Seal other end of column with frit, wash packed column with MeOH prior to use.

E. PROCEDURE

1. Instrument Operating Parameters

(a) Column temperature: 30 °C recommended, but ambient is optional.

(b) LC Mobile Phase: (45+55), Acetonitrile/Acetate buffer, 0.1M, pH 4.5, 0.005M p-TSA.

(c) Mobile Phase Flow: 1 mL/minute (95-100kPa, 1368-1440 psi).

(d) Detector: UV/VIS detector.- Shimadzu Ultraviolet-Visible spectrophotometric detector, SPD-6AV module, cell volume, 8µL; light source, tungsten halogen (WI) lamp(370-700nm); wavelength, 618nm; absorbance range, 0.005 AUFS.

(e) Post Column Reactor: PbO_2, 10% with Celite (w/w). Connect between analytical column and detector. Replace as needed to meet system suitability (E.2.a.).

(f) Total run time: 18 minutes.

2. System Suitability

(a) PbO_2 column conversion efficiency: Inject 100µL of Mixed LC working standard solution (50ng each/mL, equivalent to 20 ppb each). LMG peak height should be >90% of MG peak height for the equivalent weight (ng) injected. If this test fails, it indicates that the PbO_2 has been exhausted and the reactor column should be reprepared. The reactor column color will change from grey to white.

(b) Minimum instrument response: the signal-to-noise ratio for peaks in a 100 µL injection of the 1 ppb standard should be at least 5:1.

(c) Five replicate 100 µL injections of 50 ng/mL mixed LC working standard should have a RSD ≤ 5% in peak height and ≤2% in retention time..

(d) Chromatographic resolution should be more than 3.4 between MG and LMG based on a 100 µL injection from Mixed LC working standard.

(e) Generate a 5 point standard curve. Make duplicate 100uL injections of each Mixed LC working standard solution using specified conditions.

(f) Standard curve should give a correlation coefficient not less than 0.995.

3. Sample Preparation

Homogenize frozen catfish fillets without skin in Waring Blender. Keep tissue as cold as possible. Store homogenate in Whirl-Pak bag. Seal bag and keep tissue frozen(-70°C) until analysis.

4. Extraction of Samples

(a) Accurately weigh 10g±0.1g prepared tissue into 200mL centrifuge tube. Let stand 15 min to warm up. To fortify at 2.5, 5, or 10 ppb each of MG and LMG, add 25, 50, or 100µL Fortification Standard Mix (D.2.(p)). If fortifying, add fortification solution directly on top of tissue, then let stand 15 min more. Six samples can be conveniently processed in one day.

(b) Add 3mL hydroxylamine HCl (0.25g/mL) to all samples. Add 5mL 0.05M p-TSA to all samples. Add 10mL acetate buffer to all samples.

(c) Blend 30 seconds at medium speed with tissuemizer. Rinse probe with 6mL water, collecting rinse in 200ml tube. Use a clean probe for each sample. If availabilty of probes is limited, clean probe with acetate buffer and acetonitrile before blending next sample.

(d) Add 50mL acetonitrile and shake vigorously 30 seconds.

(e) Add 18-20 grams of alumina, cap and shake vigorously 30 seconds and centrifuge at 4°C for 10 minutes at 2500 rpm (1600 *g* RCF).

(f) Decant supernate into 250mL sep. funnel containing 100mL water and 2ml DEG.

(g) Add a second 50mL acetonitrile to 200 mL centrifuge tube containing sample, alumina and catfish plug. Cap and shake vigorously 30 seconds to break up plug.

(h) Centrifuge at 4°C for 10 minutes at 2500 rpm (1600 *g* RCF).

(i) Decant supernate into original 250mL separatory funnel containing 100mL water and 2ml DEG. Proceed immediately to next step.

(j) Add 50mL methylene chloride (CH_2Cl_2) to separatory funnel. Rotate funnel in bicycle motion (with both hands, grasp top and bottom of separatory funnel in horizontal position. Motion is as if rotating bicycle pedals, one in each hand) for 30 seconds. Do not rotate too gently or phases will not mix well and recovery will fall.

(k) Allow phases to separate (=>15 minutes). Drain lower layer into 300mL pear-shaped boiling flask. Rinse tip of separatory funnel with 2mL CH_2Cl_2, collecting rinse in flask. Place flask in 600mL beaker for support.

(l) Repeat extraction of aqueous phase in separatory funnel with 50mL CH_2Cl_2 as before and combine CH_2Cl_2 in 300mL boiling flask.

(m) Rotoevaporate contents to approximately 10mL with water bath at 50°C. Complete this step in 15 min or less. This is a convenient time to prepare the SPE tubes.

(n) Wash flask walls with 15mL CH_2Cl_2. Rotoevaporate to approximately 10mL.

(o) Add 10mL CH_3CN and rotoevaporate to about 1mL.

(p) Add 5mL CH_3CN, stopper and swirl to dissolve residue and proceed to SPE columns. *Stopping Point - Securely sealed solution may be stored in freezer (-4°C) after this step. Bring to room temperature before going to step (s).*

(q) Prewet a disposable ALN-SPE alumina column and a PRS-SPE column each with 5mL CH_3CN.

(r) Add 5mL CH_3CN to PRS-SPE and place ALN-SPE column on top of PRS-SPE column by connecting with adaptor and connect to vacuum.

(s) Transfer sample solution to ALN/PRS columns. Pass solution through ALN-SPE column onto PRS-SPE column at a drop rate of approximately 4mL/min. Each

sample may be applied to the SPE columns as the next sample is rotoevaporated. If flow is reduced by clogging, apply additional pressure with plunger at top of reservoir column to maintain flow.

(t) Rinse flask with two 5mL portions of CH_3CN and pass each wash through columns.

(u) Wash columns with 5mL CH_3CN. Draw all liquid from PRS column, just until air appears at bottom of column packing. Discard all eluates to this point.

(v) Disconnect SPE columns from each other and from vacuum. Elute PRS-SPE column by gravity into 15mL centrifuge tube with two 1mL portions of mobile phase followed by 1mL HAH/MeOH/p-TSA (2.5mg/mL). Elution should take about 15 min.

(w) With hand bulb or syringe, blow out remaining solvent from column into 15mL centrifuge tube.

(x) Dilute to 4 mL with water. *Stopping Point - Securely stoppered solution may be stored in freezer (-4°C) after this step. Remember to bring to room temperature before analysis.*

5. Instrument Analysis of Samples

Inject all five mixed LC Working Solutions, then the set of sample extracts. Follow with an injection of the 5ppb standard to verify instrument performance. Inject 100µL into LC (samples and standards). Dilute sample extracts with mobile phase if necessary, so that the peak response of analytes lies between highest and lowest standard.

6. Calculations

Prepare a standard curve. Compute the linear regression data based on peak height response for every standard. Determine the amount of analyte in the sample using standard curve equation of the form $y = mx + b$. Solve for $x = (y - b) / m$ where:

y = peak response (mm or height counts) of analyte in the unknown
b = the y axis intercept (mm or height counts)
m = slope of the line (mm/ng or height counts/ng)
x = ng of the analyte in the sample volume injected

Convert to ng/g (ppb) as follows:

$$ppb = \frac{x \left(\frac{DV(mL)}{IV(mL)}\right)}{W(g)}$$

where:

x = ng of the analyte in the sample volume injected, as calculated above
DV= Dilution volume of sample solution, i.e., 4mL for each 10g sample. High level samples may be diluted further to bring into range of standard curve. For example, if 2mL of final solution are diluted to 10mL the dilution volume would be 4mL x (10mL/2mL) = 20mL.
IV = Injection volume of sample solution in milliliters(mL),i.e. 100uL = 0.1mL
W(g) = Weight of catfish sample in grams.

G. NOTES

1. The analyst should not be overly deliberate in conducting the first steps of the extraction. Aim to carry six samples from starting step (b) to the end of step (e) within 30 min. Work as quickly as possible from starting step (b) to the end of step (i).

2. Catfish fillets should be stored in freezer (-70°C) until sample preparation and analysis.

3. This procedure will generate waste streams of organic solvents (acetonitrile, methylene chloride and methanol), aqueous solutions (acetate buffer from mobile phase) and solid waste (spent PbO_2 post-column material). All should be handled, stored and disposed of in accordance to your local Hazardous Waste Management Program protocol.

4. A linearity check was performed using mixed standard solutions and a freshly prepared PbO_2 post-column. Regression analysis, Table 1, of the standard curve data using peak area indicated correlation coefficients for MG and LMG of 0.9999 and 0.9996, respectively. Regression analysis of peak height measurements is shown in Table 4. As can be seen from the data, the LC conditions established provide a useful and linear range for MG and LMG of 4-127ng/mL and 5-149ng/mL, respectively. These concentrations representing levels of MG and LMG in catfish tissue of 1.2-38.1ppb and 1.5-44.7ppb, respectively.

5. Recovery of MG/LMG is shown, Table 2. Fortification was performed using a mixed standard, MG/LMG. No individual analyte recoveries were run. Typical %recoveries and %RSD for MG and LMG were 73, 5 and 83, 7, respectively.

6. Isolation of both MG and LMG was achieved on a median range cation exchange solid phase extraction column, propylsulfonic acid (PRS). Both compounds are completely retained and efficiently eluted. Recovery of MG/LMG without matrix (standard solution) from the PRS-SPE was 103% and 98.1%, respectively. When MG/LMG was added to tissue matrix, applied to the PRS-SPE and eluted, the average recoveries were 74.2% (%RSD=8.53, n=6) and 88.7% (%RSD=7.80, n=6), respectively.

7. The method was applied to MG-incurred catfish at various depuration times. The average levels of residual MG and LMG in these samples are shown in Table 3.

8. Retention times for MG and LMG are approximately 14 and 6 minutes, respectively. The use of the post column tends to cause some band broadening due to the increase in chromatographic volume. Since the two analytes are well resolved it does not pose any great concern. It should be noted that the demethylated products of MG/LMG may appear in the chromatograms of incurred tissue (Figure 2).

H. ACKNOWLEDGEMENTS

The authors wish to thank Dr. Steven Plakas, Food and Drug Administration, Gulf Coast Seafood Laboratory, Dauphin Island, for providing the fish tissue in support of this project.

I. REFERENCES

1. Green, F.J. (1990), *The Sigma-Aldrich Handbook of Stains, Dyes and Indicators*, Aldrich Chemical Company,Inc.

2. Muir, J.F.and Roberts, R.J. (1988), *Recent Advances in Aquaculture*, Vol. 3, Chapter 1, Croom Helm Ltd,. London/Sydney.

3. Clifton-Hadley, R.S. and Alderman, D.J., J. Fish Dis., 10:101-107(1987).

4. Bauer, K., Dangschat, H., Knoeppler, H.O. and Neudegger, J. (1988), Arch. Lebensmittelhyg., 39(4):97-102.

5. Allen, J.L. and Meinertz, J.R. (1991), J. Chromatogr., 536:217-222.

6. Rushing, L.G. and Bowman, M.C. (1980), J. Chromatogr. Sci., 18:224-232.

7. Abidi, S.L. (1983), J. Chromatogr., 255:101-114.

8. Roybal, J.E., Munns, R.K., Hurlbut, J.A. and Shimoda, W. (1989), J. Chromatogr., 467:259-266.

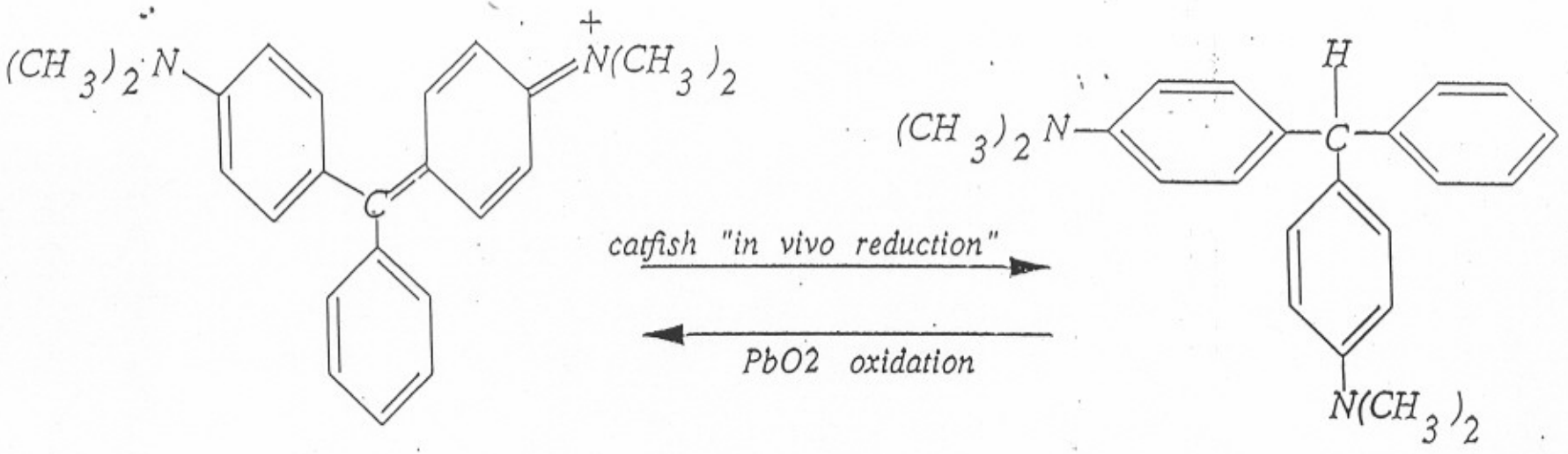
9. Allen, J.L., Gofus, J.E. and Meinertz, J.R., *Determination of Malachite Green Residues in the Eggs , Fry and Adult Muscle Tissue of Rainbow Trout (Oncorhynchus mykiss).* with permission, private oral communication 10/19/92. In press, to be published in the JAOAC International.

10. Klein, E., Edelhaeuser,M. and Lippold, R., Dtsch. Lebensm. Rundsch. (1991), 87:350-353.

J. FIGURES

1. Metabolic scheme of malachite green administered to catfish and corresponding PbO_2 post-column oxidation conversion of LMG to the chromophoric MG.

2. Typical LC/VIS chromatograms. (A) Control catfish; (B) 10 ppb fortified catfish, 5 ppb each LMG + MG; (C) residue-incurred catfish measured at 9 ppb total LMG + MG; (D) 10 ppb standard, 5 ppb each LMG + MG.



MALACHITE GREEN

LEUCOMALACHITE GREEN

Figure 1: Metabolic scheme of Malachite Green when administered to catfish and corresponding PbO_2 post column oxidation conversion of LMG to the chromophoric MG.

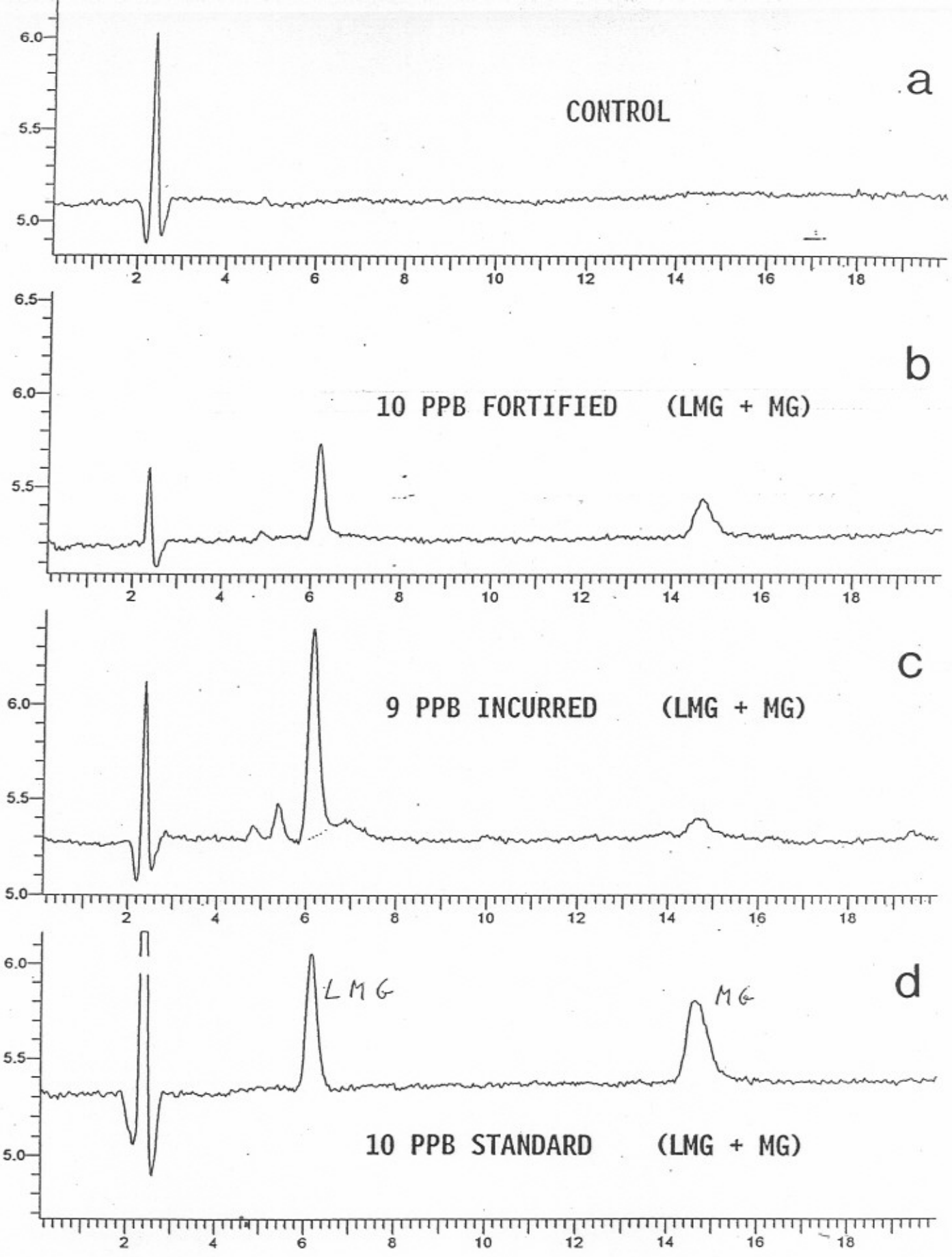

a
CONTROL
b
10 PPB FORTIFIED (LMG + MG)
c
9 PPB INCURRED (LMG + MG)
d
LMG
MG
10 PPB STANDARD (LMG + MG)

TABLE 1

Linear Regression Data, (y = mx + b) and correlation coefficients (r).

	m (area/ng)	b	r
Malachite Green (MG)	218720	2270	0.9999
Leucomalachite Green (LMG)	162820	4621	0.9996

TABLE 2.

RECOVERY (%) OF MALACHITE GREEN/LEUCOMALACHITE FROM CATFISH

MALACHITE GREEN

FORTIFICATION LEVEL, ppb	23	11	5.7
AVERAGE, %	72.9[a]	75.5[b]	69.6[c]
SD	1.40	5.17	4.82
% RSD	1.92	6.85	6.93

LEUCOMALACHITE GREEN

FORTIFICATION LEVEL, ppb	21	10	5.3
AVERAGE, %	87.4[a]	88.1[b]	82.6[c]
SD	2.55	5.23	9.49
% RSD	2.92	5.94	11.5

a - 6 determinations
b - 12 determinations
c - 10 determinations

TABLE 3.

DETERMINATION OF INCURRED MALACHITE GREEN RESIDUES (ppb) IN CATFISH (Ictalurus punctatus) TISSUE

	MALACHITE GREEN			LEUCOMALACHITE GREEN		
Sample ID	Found (ppb)[A]	SD	%RSD	Found (ppb)[A]	SD	%RSD
1	486	23.4	4.81	632	23.6	3.73
2	190	18.8	9.89	703	30.8	4.38
3	187	23.7	12.7	748	30.0	4.01
4	111	12.8	11.5	450	30.7	6.82
5	73.4	7.54	10.3	289	19.8	6.85

A - Average of four determinations each sample

TABLE 4

Linear Regression Data, (y = mx + b) and correlation coefficients (r).
m = peak height response in mm/nanograms analyte

	m	b	r
Malachite Green (MG)	54.9	0.300	0.9995
Leucomalachite Green (LMG)	70.2	-0.360	0.9981